



RESEARCH ARTICLE



Characterization of *Achlya americana* and *A. bisexualis* (Saprolegniales, Oomycota) Isolated from Freshwater Environments in Korea

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ABSTRACT

Many members of the Saprolegniales (Oomycete) cause mycoses and disorders of fishes, of which *Achlya* and *Saprolegnia* are most ubiquitous genera worldwide. During a survey of the diversity of freshwater oomycetes in Korea, we collected seven isolates of *Achlya*, for which morphological and molecular phylogenetic analyses enabled them to identify as *Achlya americana* and *Achlya bisexualis*. In Korea, only a species of *Achlya*, *A. prolifera*, has been previously found to cause seedling rot on rice (*Oryza sativa*), but none of the two species have been reported yet. Importantly, *A. bisexualis* was isolated from a live fish, namely rice fish (*Oryzias latipes*), as well as freshwater, and this is the first report of *Achlya*-causing mycoses on freshwater fishes in Korea. The presence of *A. americana* and *A. bisexualis* on live fish in Korea should be closely monitored, as considering the well-known broad infectivity of these species it has the potential to cause an important emerging disease on aquaculture industry.

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1. Introduction

The kingdom Straminipila is a remarkably diverse group that includes oomycetes, brown algae, and planktonic diatoms. The oomycetes have previously been classified under the kingdom Fungi, with which they share several convergent traits, such as osmotrophic nutrition and mycelial growth [1], thus often called “fungus-like organism” or “pseudo-fungi.” These organisms have evolved both pathogenic and saprophytic lifestyles. The largest subclass Peronosporomycetidae is mostly parasitic on plants [2], the vast majority of which was found on the order Peronosporales (includes *Peronospora*, *Phytophthora*, and *Pythium*), which occur important diseases on crops, ornamental plants, and forest trees [3]. The second subclass Saprolegniomycetidae more depends on water, thus often called “water moulds.” The largest order Saprolegniales, in particular members of *Achlya* (Achlyaceae), *Aphanomyces* (Verrucalvaceae), and *Saprolegnia* (Saprolegniaceae), are responsible for significant infections on fishes in nature and aquaculture, although many members still behave as saprophytes on plants and animal debris.

The family Achlyaceae has been introduced for accommodating *Achlya* s. str., *Brevilegnia*, *Dictyuchus*, and *Thraustotheca* [4], all of which are

characterized by the presence of three different types of zoospore discharge, namely achlyoid, dictyucoid, and thraustothecoid, at asexual reproduction, but also at sexual reproduction, by one to more oospores per oogonium that are predominantly [4]. Before the replacement to this family, the genus *Achlya* was classified under the family Saprolegniaceae [5,6]. In members of this genus, primary aplanospores are discharged from sporangium, and before swimming away, stay near the exit of the sporangium until a ball of spores is formed [4,7]. Molecular phylogenetic studies have shown that *Achlya* s. lat. is polyphyletic [8–10], and for a group of *Achlya* species with subcentric oospores, a new genus *Newbya* has been described by Spencer et al. [10]. As a result, Rocha et al. [7] have transferred *Achlya androgyna* to *Newbya*. To date, approximately 80 species have been accepted in the genus *Achlya* (www.indexfungorum.org; [7]). About 20 species of *Achlya* have previously been reported to infect live fishes in many countries [11,12]. Thus far, in Korea, there was no report of mycoses caused by *Achlya* species on live fishes, although only a species of *Achlya*, *A. prolifera*, has previously been found to cause seedling rot on rice (*Oryza sativa*) [13].

Recent phylogenetic studies have largely been biased to the economically relevant plant pathogens

Table 1. Information of *Achlya* isolates used in this study.

<i>Achlya</i> species	Culture no.	Substrate	Geographic origin	ITS/cox2/cox1
<i>A. americana</i>	CNUFC-CPWB8-1	Fresh water	Yongbong-ro, Buk-gu, Gwangju, Korea (35°10'20.45"N 126°53'57.06"E)	MF996495/MH673798/MH673797
<i>A. americana</i>	CNUFC-CPWB8-2	Fresh water	Yongbong-ro, Buk-gu, Gwangju, Korea (35°10'20.45"N 126°53'57.06"E)	MF996496/–/–
<i>A. bisexualis</i>	CNUFC-CPWB4-1	Fresh water	Yongbong-ro, Buk-gu, Gwangju, Korea (35°10'20.45"N 126°53'57.06"E)	MF996493/MH673800/MH673799
<i>A. bisexualis</i>	CNUFC-CPWB4-2	Fresh water	Yongbong-ro, Buk-gu, Gwangju, Korea (35°10'20.45"N 126°53'57.06"E)	MF996494/–/–
<i>A. bisexualis</i>	W351 (AQPXFG0000000024)	Rice fish (<i>Oryzias sinensis</i>)	Gui-myeon, Wanju-gun, Jeollabuk-do, Korea (35°38'20"N 127°06'12"E)	MH667998/–/–
<i>A. bisexualis</i>	W352	Rice fish (<i>Oryzias sinensis</i>)	Gui-myeon, Wanju-gun, Jeollabuk-do, Korea (35°38'20"N 127°06'12"E)	MH667999/–/–
<i>A. bisexualis</i>	W413	Fresh water	Geumsan-myeon, Gimje-si, Jeollabuk-do, Korea (35°43'07"N 127°01'47"E)	MH668000/–/–

of oomycetes, mostly of Peronosporales [3,14,15], while the diversity of freshwater or marine oomycetes has been largely underestimated [16,17]. To survey the diversity of freshwater oomycetes in Korea, we collected numerous isolates from diverse freshwater environments, such as water, fishes, aquatic plants, etc. In total seven *Achlya* isolates were obtained, one which was obtained from a rice fish (*Oryzias sinensis*) with unusual behavior at the narrow lake. While it lives, the rice fish swam slowly and often upside down, and after a few minutes died, although there was no clear mycoses symptom on the surface of the rice fish. It is the aim of this study to clarify the identity of the so far unknown *Achlya* species in Korea, based on morphological and molecular analyses.

2. Materials and methods

2.1. Oomycete isolates

Freshwater oomycetes were collected from a living rice fish (*O. sinensis*) at a narrow lake in Wanju, but also from freshwater at Yeongsan River in Gwangju, Korea. The samples were collected into a 15 ml conical tube, and during moving to laboratory kept in icebox. To isolate the oomycete strains from a fish, we washed it with distilled water three times, placed on potato dextrose agar plates, and incubated at 20 °C for 2 d. For freshwater, we used a simple plating technique whereby it is distributed throughout a thin layer of the water agar plate. Hyphal tips were isolated under a microscope and transferred onto a new PDA plate. For observations of cultural and morphological characteristics, seven isolates with *Achlya*-like morphology were selected and incubated on PDA at 25 °C for 3 d. Two

representative isolates, W351 and CNUFC-CPWB8-1 have been deposited at the Culture Collection of the Nakdonggang National Institute of Biological Resources (NNIBR, Sangju, Korea) with accession numbers of AQPXFG0000000024 and NNIBRF G9371, respectively. Information on all isolates used in this study was shown in Table 1.

2.2. Morphological analysis

Morphological investigation and photographing were done using a model BX53F microscope (Olympus, Tokyo, Japan) equipped with a DigiRetina 16M digital camera (Tucsen, Fuzhou, China). Measurements were performed at 100× or 200× for hyphae and gemmae, 400× for sporangia, and 1000× for zoospores.

2.3. Molecular phylogenetic analysis

Genomic DNA was extracted from mycelia, which were harvested by forceps, using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The complete ITS rDNA region was amplified by PCR using an oomycete-specific primer set, primers ITS1-O [18] and LR-0 (reverse complementary to LR-0R [19]). For a few isolates indistinguishable on the ITS sequences, the partial *cox2* and *cox1* genes were additionally amplified as described previously [20]. Amplicons were sequenced by MacroGen Inc., Seoul, Korea. Available for *Achlya* species, all sequences of ITS rDNA were retrieved from GenBank, and in addition, a sequence of *Aphanomyces stellatus* (KP006462) was downloaded and used as outgroup. Alignments were performed using MAFFT version 7

[21], employing the Q-INS-i algorithm [22]. Phylogenetic inference was done in MEGA version 6.0 [23] for Minimum Evolution Inference, using the Tamura-Nei substitution model and performing 1000 bootstrap replicates. All other parameters were set to default values. For Maximum Likelihood (ML) analyses, 1000 rounds of random addition of sequences as well as 1000 fast bootstrap replicates were performed using RAxML version 7.0.3 [24] as implemented in raxmlGUI version 1.3 [25] using the GTRCAT variant.

3. Results

3.1. Phylogeny

The ITS rDNA sequences were used to infer the phylogenetic relationship of *Achlya* species, including seven Korean isolates and previously published authentic isolates. In the ITS tree (Figure 1), the Korean accessions were divided into two distant clades. Two isolates, CNUFC-CPWB8-1 and CNUFC-CPWB8-2, clustered within a complex group, containing five different species of *Achlya*, *A. americana*, *A. aquatica*, *A. dubia*, *A. glomerata*, and *A. piralis* (in yellow box), with maximum support in both ME and ML analyses. To further resolve this group at the species level, two mitochondrial markers, *cox2* and *cox1*, were additionally sequenced. In the *cox* tree (in green box), the isolate CNUFC-CPWB8-1 is closest to *A. americana*, with only a nucleotide substitution. The grouping was supported by moderate BS values of 75% in ME and 80% in ML analyses. Other five Korean isolates (in blue box) were identical to the reference sequences of *Achlya bisexualis* available in GenBank, and thus clustered within a clade representing *A. bisexualis*, with moderate supporting values of 79 and 84% in the two analyses. The clade further grouped with *Achlya oblongata* (ME BS of 99% and ML BS of 88%), but was distantly related to *Achlya ambisexualis* and *Achlya heterosexuales*, which were morphologically similar to *A. bisexualis*.

3.2. Taxonomic description

Achlya americana Humphrey, Transactions of the American Philosophical Society 17: 116 (1892) [MB#205422] (Figure 2).

Mycelium limited or extensive, diffuse or dense. Hyphae stout, sometimes stiff, branched. Gemmae abundant or sparse, cylindrical, fusiform, or irregular; usually terminal and single, occasionally intercalary and catenulate; discharging zoospores or germinating by a hypha in filiform gemmae. Sporangia fusiform, cylindrical or clavate, sympodially branched, 130–700 µm long, 20–50 µm wide.

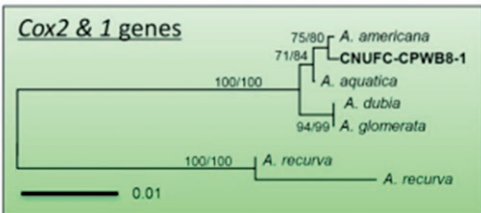
Zoospores monomorphic, discharge through an apical pore, and behavior achlyoid, zoospore cluster persisting or falling away in part from exit orifice, sometimes they germinate while still in the sporangium; primary spore cysts 10–11 µm in diameter. Oogonia lateral, occasionally terminal, rarely intercalary; spherical or obpyriform, occasionally oval, rarely dolioform (25–)50–80(–140) µm in diameter. Oogonial stalks stout, straight, slightly curved, or irregular; unbranched. Oospores eccentric, spherical, 20–30 µm in diameter, and produced in the number of 2–15(–35) per an oogonium.

Note: *Achlya americana* is a widespread species in temperate regions. Within members of *Achlya*, *A. americana* is quite easily recognizable by several prominent characteristics of the sexual structures. The oogonia were eccentric, short-stalled, and contained 5–15 oospores, but the oospores exhibited smooth surface, with conspicuous pits. All morphological characteristics are well consistent with the previous descriptions of this species [26,27].

Achlya bisexualis Coker & Couch, Journal of the Elisha Mitchell Scientific Society 42: 207 (1927) [MB#258533] (Figure 3).

Mycelium of oogonial thallus limited or extensive, often dense near substratum. Hyphae slender, branched, 10–15 µm wide. Gemmae abundant (35–)50–70(–100) µm wide (35–)60–100(–150) µm long, various in shape; spherical, pyriform, flask-shaped, subspherical, obovate, obpyriform, short-cylindrical, but also rarely filiform, with thin or thick wall; terminal or intercalary, single or catenulate; discharging zoospores or germinating by a hypha in filiform gemmae; capable as oogonia in spherical gemmae. Sporangia fusiform or filiform, infrequently cylindrical, sympodially branched or basipetalous succession, 150–400 µm long, 25–35 µm wide. Zoospores monomorphic, discharge through an apical pore, and behavior achlyoid, zoospore cluster persisting or falling away in part from exit orifice; primary spore cysts 9.0–11.5 µm in diameter. Oogonia lateral, occasionally terminal, rarely intercalary; spherical or obpyriform, occasionally oval, rarely dolioform (35–)60–80(–120) µm in diameter. Oogonial stalks stout, straight, slightly curved, or irregular; occasionally once-branched; slightly flared distally on some oogonia. Oospores not seen.

Note: All morphological characteristics are well in agreement with the descriptions of *A. bisexualis* [28,29]. In the present samples, non-maturing oospores were found, although there were abundant gemmae and oogonia, suggesting that this is a heterothallic species that it was known to form the oospheres infrequently to rarely maturing. This distinct character enables *A. bisexualis* to differentiate



from two morphologically close species, *A. ambisexualis* and *A. heterosexualis*, with the oospheres predominantly maturing. The spherical or pyriform gemmae in oogonial mycelia are another key character unique for *A. bisexualis*, different from all other species of *Achlya*, as well *A. ambisexualis* and *A. heterosexualis*, with mostly slender or clavate gemmae, as mentioned by Barksdale [29].

Aquatic oomycete infections of fish are widespread in freshwater and represent the most important disease occurring on wild and cultured fishes. Several members of the genus *Achlya* are responsible for significant infections, particularly in aquaculture facilities, out of which *A. americana* and *A.*

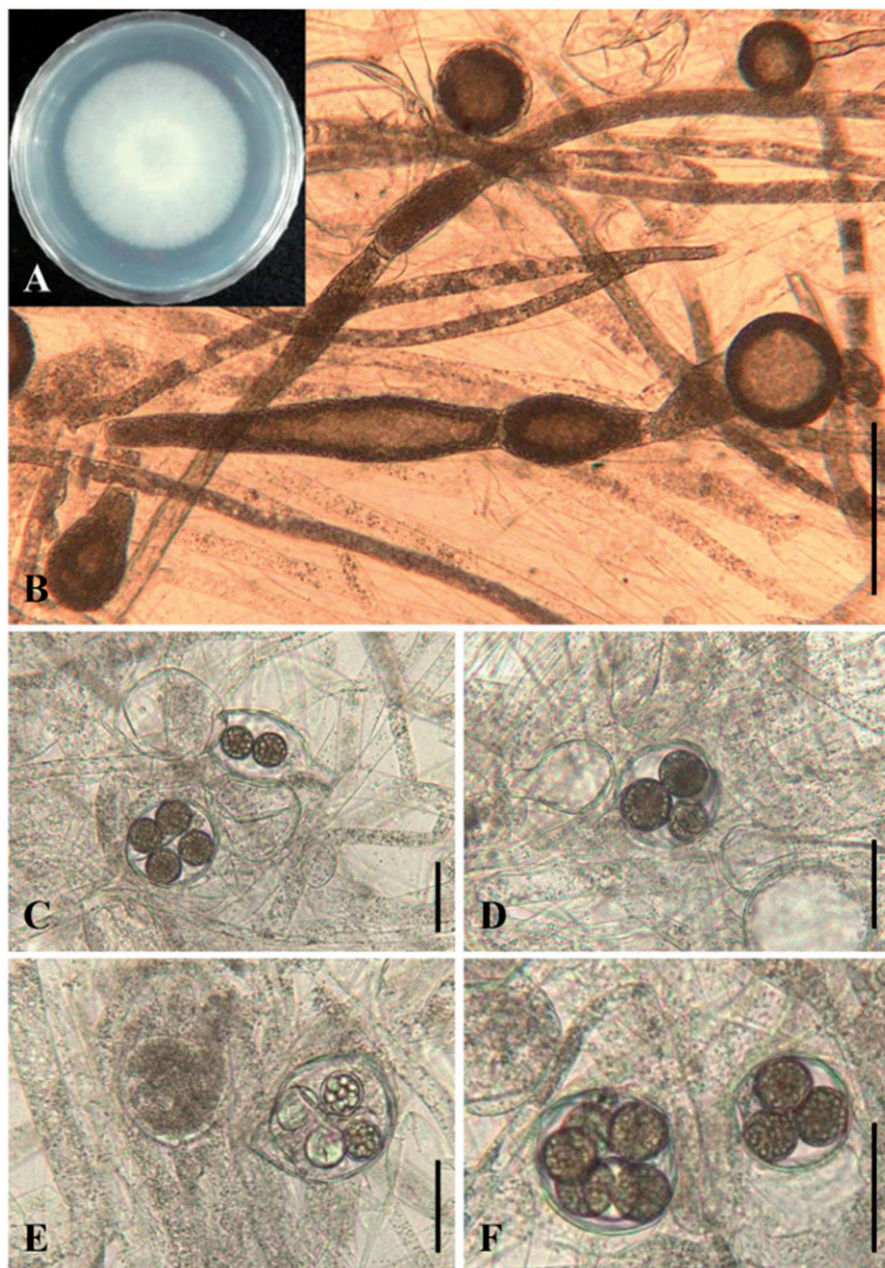


Figure 2. Cultural and morphological characteristics of *Achlya americana* CNUFC-CPWB8-1 isolated from freshwater. (A) Growth on PDA at 25°C for 3 d; (B) Sporangia and gemmae; (C–F) Oogonia and oospores. Scale bar = 100 µm for B, 50 µm for C–F.

bisexualis are of notorious pathogens. There have been previous reports that *A. americana* [30–35] and *A. bisexualis* [31,36–40] infect live fishes or their eggs, sometimes with a high level of pathogenicity [41]. *A. americana* and *A. bisexualis* are ubiquitous worldwide [11,12], but to date, there was no report of these two species in Korea. Considering that in other countries outbreaks of *Achlya* infections continue to cause a big problem in cultured fishes and fish eggs, their presences in Korea also have the potential to cause an important emerging disease on aquaculture industry. Thus, these species should be closely monitored.

Importantly, some species of *Achlya* have received considerable interest as steroid producers [42–44]. Both *A. americana* and *A. bisexualis* have

been reported to produce useful steroid hormone that serve to induce the differentiation of sex organs and to coordinate the processes leading to fertilization [44–46]. The two species also produce enzymes with excellent degradative activities against 1,3-beta-glucan, pectin, cellulose, hemicellulose, and lignin [47,48]. Especially in *A. bisexualis*, 1,3-beta-glucan is used as a major cytoplasmic reserve material [49], and thus when a carbon source, such as glucose, is no longer available, as the activity of 1,3-beta-glucosidase increases, the glucan decreases significantly [47].

Increasingly becoming intensified, poor water quality, stress of climate changes, and injuries associated with handling and grading are frequently associated with fungal outbreaks in both natural and

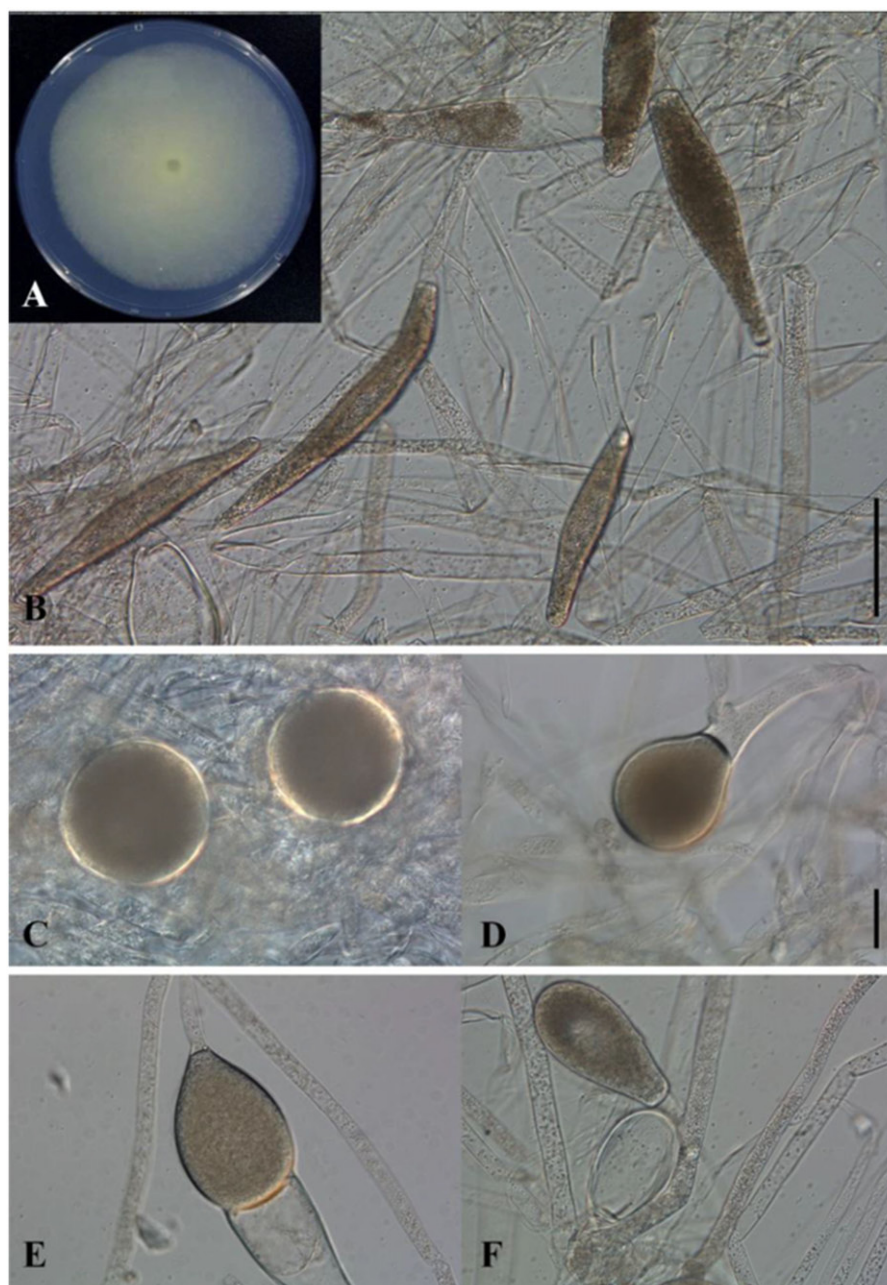


Figure 3. Cultural and morphological characteristics of *Achlya bisexualis* isolated from rice fish (*Oryza sativa*) in freshwater. (A) Growth on PDA at 25 °C for 3 d; (B) Sporangia; (C–F) various shapes of gemmae. Scale bar = 100 µm for B, 40 µm for B–F.

cultured fishes. Many control treatments have been conducted, none of which is considered as effective as chemical compounds, e.g., malachite green. Unfortunately, however, its use has been prohibited by the potential teratogenic or mutagenic properties of malachite green [50] in most countries, including Korea. Considering the lack of the efficient control treatments, thus, the rapid and precise identification tool is an essential prerequisite for preventing or limiting oomycete outbreaks, but morphology-based identification is often difficult or insufficient to discriminate between closely related species, and the sexual organs useful for species identification are rarely formed, as in *A. bisexualis*. In line with the result of Leclerc et al. [9] that molecular-based

phylogenetic analysis is useful for *Achlya* taxonomy, in this study the ITS sequences were able to distinguish most *Achlya* species, but also insufficient to delineate between closely related species, e.g., *A. americana* and allied species. As a result, in addition to the ITS rDNA [51], other gene markers with a high-resolution power, e.g., *cox2* mtDNA [20], are essential to identify *Achlya* isolates at species level and for digging in earnest on the aquatic oomycete diversity.

Disclosure statement

No potential conflict of interest was reported by the authors.

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